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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US92/09416 <b>(22) International Filing Date:</b> 3 November 1992 (03.11.92)  <b>(30) Priority data:</b> 803,192                      5 December 1991 (05.12.91)      US  <b>(71) Applicant:</b> BOARD OF REGENTS OF THE UNIVERSITY OF NEBRASKA [US/US]; Varner Hall, 3835 Hol-drege Street, Lincoln, NB 68583-0745 (US).  <b>(72) Inventor:</b> CHEN, Swei-Shen ; 3601 Cape Charles Court, Lincoln, NB 68516 (US).  <b>(74) Agent:</b> HAMRE, Curtis, B.; Merchant, Gould, Smith, Edell, Welter & Schmidt, 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402 (US).		<b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, European pa-tent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ALLERGEN SPECIFIC IMMUNOGLOBULIN-E PRODUCER TEST FOR DIAGNOSING ALLERGIES  <b>(57) Abstract</b>  The invention includes a method for detecting immunoglobulin-E (IgE) secreting cells through utilization of an Allergen Specific IgE Provocation Test (ASEPT™ test). The invention also includes methods for diagnosing allergies in a patient, even if that patient is not exhibiting allergic symptoms. The invention also includes a diagnostic kit enabling physicians and re-searchers to diagnose allergies. The kit includes solid carriers with allergens immobilized thereon, detecting reagents and an indicator with standardized results.		

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ALLERGEN SPECIFIC IMMUNOGLOBULIN-E PRODUCER TEST  
FOR DIAGNOSING ALLERGIES

5

BACKGROUND OF THE INVENTION

Millions of people throughout the world suffer from allergies caused by their exposure to allergens. Such an allergenically reactive person will have B-  
10 lymphocytes that will produce specific immunoglobulin-E (IgE) antibodies to the specific allergen. The IgE antibodies produced specifically in response to the allergen are then strongly attracted to mast cells and basophils. The IgE antibodies become bound to the  
15 surfaces of the mast cells and basophils. When these basophil and mast cells are subsequently exposed to allergens, these allergens attach to the IgE antibodies bound to the cellular surfaces.

After repeated exposure to the allergen, these  
20 basophils and mast cells undergo a process of degranulation wherein the contents of the intracellular granules of the mast cells and basophils are released from the cell. These intracellular granules include histamines, serotonin, and leukotrienes which cause  
25 inflammation, itching of the eyes and throat, sneezing, post nasal drip, and/or congestion in patients with rhinitis. In some severe asthmatic cases, the allergic response may include constriction of bronchiole muscles and anaphylactic shock.

30 Physicians generally use a skin test to diagnose allergies. This test involves taking small quantities of allergen in a diluted extract and introducing these allergens into the skin by pricking the skin with a needle which has been dipped in the allergen extract. A  
35 typical skin test may require more than 40 separate needle pricks to test the effects of various allergens to the patient in vivo. A raised, red bump in a given skin test area indicates the patient has produced IgE antibodies specific to the allergen injected into the  
40 skin.

There are several disadvantages with the standard skin test. Some patients, especially young children, do not tolerate skin tests very well. In rare cases, a skin test may result in a severe allergic reaction in the patient. Even those patients that are able to tolerate these skin tests will experience some discomfort and pain as a result of the pricking and inflammation associated with the test. Other allergy tests for detecting the presence of IgE do exist. A radioallergosorbent test (RAST) or a paper radioimmunosorbent test (PRIST) may be performed, in which the patient's blood serum is tested for the presence of a specific IgE antibody. Both these tests, however, are not ex vivo provocation tests. Instead, these tests merely detect whether IgE is present. In addition, the PRIST test is subject to modulation by the level of lymphokines and infections present in the patient. The RAST test produces false negative reactions when  $1.0 \times 10^4$  to  $1.0 \times 10^5$  fold molar excess of competing immunoglobulins are normally present.

Therefore, it is an object of the invention to provide a method for detecting IgE producing cells by utilizing an allergen-specific IgE producer test (ASEPT<sup>TM</sup> test). This method permits detection of IgE without interference from concomitant competing immunoglobulin secreting cells. Another object is to provide a method of diagnosing specific allergies through use of ASEPT<sup>TM</sup> test. It is a further object of this invention to provide a method of diagnosing allergies in a patient who is not exhibiting allergic symptoms by inducing the production of IgE through an ex vivo provocation test utilizing ASEPT<sup>TM</sup> test. The ASEPT<sup>TM</sup> ex vivo provocation test also permits a physician to assess the efficacy of immuno-therapy or diagnose allergies without subjecting the patient to a possible severe reaction. Yet another object is to provide a test kit for diagnosing specific allergies in a patient.

SUMMARY OF THE INVENTION

According to one aspect of the invention there is provided a method for detecting immunoglobulin-E secreting cells. This method involves applying immunoglobulin secreting cells onto a solid carrier which has concentrated amounts of one or more different types of allergens bound to its surface. The number of immunoglobulin secreting cells must be applied in sufficient amounts to ensure that some of the cells delivered onto the solid carrier are potentially immunoglobulin-E secreting cells.

These immunoglobulin secreting cells are then incubated on the solid carrier for a sufficient amount of time to allow these cells to secrete immunoglobulins onto the solid carrier. As these cells secrete immunoglobulins, concentration gradients of immunoglobulin form around each immunoglobulin secreting cell as the immunoglobulins bind to the allergens immobilized on the solid carrier. Developing agents which can detect the presence of immunoglobulin-E on the solid carrier may be applied to the solid carrier. The detected areas indicate that immunoglobulin-E is present on the solid carrier.

Another aspect of the invention involves a method for diagnosis of allergies. This method is similar to the method of detecting immunoglobulin-E as discussed above with the exception that the detected areas are quantified to ascertain whether an allergic reaction has occurred. The detected areas are counted and compared to standardized results for given amounts of peripheral blood leukocytes added to a solid carrier.

A further aspect of the invention is a modification to the method for diagnosis of allergies. This method involves diagnosing allergies in a person who is not exhibiting allergic symptoms. This method is known as an ex vivo provocation test in which peripheral blood



leukocytes from a patient are applied to the solid carrier. These peripheral blood leukocytes are incubated on the solid carrier for an extended period of time to allow these cells to transform into immunoglobulin secreting cells and produce detectable amounts of immunoglobulin. This method of diagnosis also permits a physician to ascertain if a patient is allergic to certain allergens without performing an in vivo test, like the skin test, which may subject the patient to a severe allergic reaction. This method of diagnosis also permits a physician to assess the efficacy of immunotherapy without any risk of side effects to the patient.

A final aspect of the invention involves a diagnostic test kit for detecting the presence of allergies in a patient. The kit includes at least one solid carrier with at least one type of allergen immobilized thereon. Preferably, the solid carrier has a plurality of wells on the solid carrier, each well having different types of allergens bound thereto so that the patient may be tested against a variety of allergens. The kit further includes diagnosing reagents which detect allergen specific immunoglobulin-E bound to the solid carrier. An indicator, such as a card, is also included in the kit so that the detected areas of allergen-specific immunoglobulin-E may be compared to standardized results.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph depicting developed complexes where IgE has been bound to the solid carrier. The solid carrier in Fig. 1(1) has been coated with BF 815 and the cells have been stimulated with IL-4 at 250 U/ml. b-GAME. The solid carrier in Fig. 1(2) has been coated with keyhole limp t hemocyanin (KLH). The solid carrier in Fig. 1(3) is a blank control. The solid carrier in Fig. 1(4) has been coated with KLH and the



cells have been stimulated with KLH at 1  $\mu$ G/ml. b-BF 815.

Figure 2 is a dose-response curve of an ELISA-plaque test in antigen-stimulated in vitro cultures where  $5 \times 10^6$  spleen cells were subjected to various levels of soluble KLH in vitro. The graph illustrates the dosage of KLH versus the number of plaques representing sites of IgE production for  $5 \times 10^6$  spleen cells applied to the solid carrier.

Figure 3 depicts the kinetics of the ELISA-plaque test in antigen-stimulated in vitro cultures. Graphs A and B illustrate the type of antigen stimulation (KLH, IL4+LPS, or background) on the solid carrier versus the number of plaques representing sites of IgE production for  $5 \times 10^6$  spleen cells applied to the solid carrier.

Figure 4 depicts a bar graph illustrating dose responses of soluble phospholipase in vitro versus the number of plaques representing sites of IgE produced for  $5 \times 10^6$  spleen cells applied to the solid carrier.

Figure 5 depicts a bar graph illustrating the amount of soluble ovalbumin stimulation in vivo versus the number of plaques representing sites of IgE produced for  $5 \times 10^6$  spleen cells applied to the solid carrier.

Figure 6 depicts a bar graph illustrating the number of plaques formed in different lymphoid tissues when these cells are challenged with Sendai virus.

#### DETAILED DESCRIPTION OF THE INVENTION

Until the present invention, physicians and researchers primarily used the skin test to diagnose allergies. The skin test is an in vivo immunoglobulin-E (IgE) provocation test which is laborious, uncomfortable to the patient, and may cause systemic reactivities in the patient. According to the present invention, a physician can utilize an ex vivo IgE test which permits specific diagnosis of a patient's allergies. This test is known as the allergen-specific IgE producer test

(ASEPT™ test). The ASEPT™ test permits a physician to take a patient's cells and contact them with a suspected allergen ex vivo. Thus, the associated risks with the skin test are eliminated. If the patient carries the allergy, use of the ASEPT™ test will provoke the patient's cells to produce IgE specific to the allergen. The physician then has identified an allergen to which the patient is allergic.

The present invention detects IgE secreting cells in an allergic patient through a multi-step procedure. Blood is taken from a patient suspected of having allergies. The blood is processed using well-known techniques such as Ficoll-Hypaque centrifugation to isolate peripheral blood leukocytes. Approximately 10% of the peripheral blood leukocytes are immunoglobulin secreting cells during an active phase of an allergic reaction. Each immunoglobulin secreting cell produces a specific immunoglobulin such as immunoglobulin-A (IgA), immunoglobulin-G (IgG), immunoglobulin-M (IgM), immunoglobulin-E (IgE), and the like. These immunoglobulin secreting cells are resuspended in biocompatible aqueous solutions which permit the cells to biologically function as they did in the patient's body. This immunoglobulin secreting cell solution may then be used in various concentrations, preferably in 1.0 ml aliquots.

The immunoglobulin secreting cell solution is then applied to a solid carrier having concentrated amounts of one or more types of allergens immobilized thereon. It is important that the number of immunoglobulin secreting cells is sufficient to deliver immunoglobulin-E secreting cells to the solid carrier because immunoglobulin secreting cells producing IgG, IgA and IgM combined outnumber IgE-secreting cells by approximately 1000 fold. Therefore, it is necessary that at least about  $1.0 \times 10^4$  immunoglobulin secreting cells are applied to the solid carrier to ensure that

some of the immunoglobulin secreting cells are IgE secreting cells. Preferably, the immunoglobulin secreting cell solution is applied to the solid carrier in 1.0 ml aliquots such that at least about  $1.0 \times 10^5$  peripheral blood leukocytes/ml are applied to the solid carrier, more preferably about  $1.0 \times 10^5$  peripheral blood leukocytes/ml to about  $5.0 \times 10^7$  peripheral blood leukocytes/ml are applied to the solid carrier, and most preferably, about  $1.0 \times 10^6$  peripheral blood leukocytes/ml to about  $1.0 \times 10^7$  peripheral blood leukocytes/ml are applied to the solid carrier. This ensures that at least about  $1.0 \times 10^4$  immunoglobulin secreting cells/ml are applied to the solid carrier, more preferably about  $1.0 \times 10^4$  immunoglobulin secreting cells/ml to about  $5.0 \times 10^6$  immunoglobulin secreting cells, and most preferably, about  $1.0 \times 10^5$  immunoglobulin secreting cells/ml to about  $1.0 \times 10^6$  immunoglobulin secreting cells/ml are applied to the solid carrier.

Preferably, the solid carrier has one or more wells, each having a surface area of at least about  $1.5 \text{ cm}^2$  and preferably about 1.5 to about  $1.76 \text{ cm}^2$  when 1.0 ml aliquots of the immunoglobulin secreting solution are applied. The surface of the solid carrier need only be about  $0.33 \text{ cm}^2$  to about  $1.76 \text{ cm}^2$  when 0.25 ml aliquots of the immunoglobulin secreting solution are applied. The range of immunoglobulin secreting cells per ml noted above and the surface area of the solid carrier allow the immunoglobulin secreting cells to be spatially-separated from each other. The term spatially-separated means that the immunoglobulin secreting cells form a single cell layer on the solid carrier such that each immunoglobulin secreting cell can contact the solid carrier without substantial interference from other immunoglobulin secreting cells. By ensuring that the immunoglobulin secreting cells are spatially-separated,

the doctor or researcher will be able to identify any immunoglobulin-E secreting cells that may be present.

The solid carrier can be made from any substance which immobilizes a sufficient amount of allergen which is capable of binding IgE. The solid carriers may be made from, but not limited to, polystyrene, nitrocellulose or Teflon® membranes.

Allergens may be fixed to the solid carrier such as the polystyrene or nitrocellulose membranes by incubating those membranes in an aqueous solution containing the specific allergens to be tested. The allergens may also be covalently linked to a solid surface such as a Teflon® membrane (produced by Millipore as Durapore, IMMOBILON AB). These solid carrier membranes should immobilize at least about 1.0  $\mu\text{g}$  allergen/ $\text{cm}^2$  to permit subsequent binding of the immunoglobulins to the immobilized allergen. Preferably, the solid carrier membranes immobilize about 1-100  $\mu\text{g}$  of allergen per  $\text{cm}^2$ , and most preferably about 10-50  $\mu\text{g}$  of allergen/ $\text{cm}^2$ .

Any type of allergen can be utilized on the solid carrier. The type of allergen tested for may include, but not be limited to, microbial, flora, fauna and chemical allergens. For example, a physician or researcher may test for a patient's allergic reaction to any of the common allergens as disclosed by Samter et al., Immunological Diseases, 4th Edition, Vol. II, 989-1001, 1988, which is incorporated herein by reference. A doctor may test more than one type of allergen immobilized on the solid carrier so long as each allergen has a concentration of at least about 1.0  $\mu\text{g}/\text{cm}^2$  on the solid carrier. Preferably, not more than four different types of allergens are immobilized on any one given test area of the solid carrier.

After the immunoglobulin secreting cells have been applied to the solid carrier, they are resuspended in tissue culture medium containing 5% fetal calf serum and

then incubated at least at about room temperature on the solid carrier for a sufficient amount of time to permit the immunoglobulin secreting cells to produce immunoglobulins. Preferably, the immunoglobulin secreting cells are incubated at about 37°C in a plastic module containing about 5 to about 10% CO<sub>2</sub>. The immunoglobulin secreting cells should be incubated at least about two hours to allow these cells to produce detectable amounts of immunoglobulin. Preferably, the immunoglobulin secreting cells are incubated on the solid carrier about 2-6 hours.

During the incubation step, substantially all of the allergen-specific immunoglobulin secreting cells produce immunoglobulins which bind to the allergens immobilized on the solid carrier. As the immunoglobulins are produced, concentration gradients form around the immunoglobulin secreting cell as the immunoglobulins become bound to the immobilized allergens in the vicinity of the specific immunoglobulin secreting cell. The immobilized allergens and immunoglobulin secreted from these cells form allergen/immunoglobulin complexes. Some of the immunoglobulin secreting cells may produce IgE should the patient be sensitive to one of the types of allergens found on the solid carrier. As a result, the IgE produced in response to the specific allergen will bind to that allergen to form a allergen/allergen-specific IgE complex. The term allergen-specific IgE refers to the IgE produced by the immunoglobulin secreting cell in direct response to the specific allergen or allergens immobilized on the solid carrier.

The number of immunoglobulin secreting cells producing IgE in specific response to the allergen depends on the patient's sensitivity to the allergen on the solid carrier. The more sensitive the patient is to the allergen, the more IgE immunoglobulin secreting cells the patient will produce. It is important to note



that even though IgG and IgA immunoglobulin secreting cells are present in hundreds to a thousand fold higher frequencies, their secreted immunoglobulins are readily captured and immobilized by the allergen-coated solid carrier and are unable to compete and displace allergen-specific IgE bound to the solid carrier at a distance.

Following the incubation of the immunoglobulin secreting cells on the solid carrier, developing agents capable of detecting the allergen/allergen-specific immunoglobulin-E complexes are combined on the solid carrier. The developing agents are capable of producing a developed complex where allergen-specific immunoglobulin-E has bound to the specific allergen on the solid carrier to form the allergen/allergen-specific immunoglobulin-E complex. The physician or researcher may then determine the presence of the allergen-specific IgE on the solid carrier by counting the developed complexes that have been detected on the solid carrier.

The physician or researcher may utilize numerous detecting techniques including Radioimmunoassay (RIA) (which utilizes radiolabeled ligands specific for the allergen-specific IgE produced). In addition, the technique of Fluorescence Immunoassay (FIA) could be used to develop the allergen-specific IgE found on the solid carrier. When utilizing FIA, a physician or researcher combines a fluorescent ligand specific for the allergen-specific IgE on the solid phase. Depending on the FIA technique used (Fluorescence Quenching, Fluorescence Enhancement or Fluorescence Polarization) an increase or decrease in fluorescence will reveal the presence of the allergen-specific IgE on the solid carrier.

In a preferred embodiment, a physician or researcher can utilize the Enzyme Linked Immunosorbent Assay (ELISA) as a developing agent. In a most preferred embodiment an allergen specific IgE producer test (ASEPT™ test) is utilized which can detect the

production of IgE ex vivo. The ASEPT™ test technique can utilize a developing agent such as an effective amount of a biotinylated anti-human IgE conjugate which may be applied onto the solid carrier for an effective amount of time to permit the biotinylated anti-human IgE conjugate to bind to the allergen/allergen-specific IgE complex to form an intermediate complex. An effective amount of biotinylated anti-human IgE conjugate is at least about 0.25  $\mu\text{g}$  of the biotinylated conjugate per ml of substrate buffer solution, preferably between 1 to 10  $\mu\text{g}$  of the biotinylated conjugate per ml of the phosphate buffer solution. An effective amount of time to apply the biotinylated anti-human IgE conjugate onto the solid carrier is at least about 10 minutes, preferably about 45 minutes to 1 hour.

After the biotinylated anti-human IgE conjugate is applied to the solid carrier for an effective amount of time, the solid carrier may be washed with a phosphate buffer solution to remove the unreacted biotinylated anti-human IgE conjugate. An effective amount of a streptavidin enzyme complex is then applied to the solid carrier for an effective amount of time to permit the streptavidin/enzyme complex to bind to the intermediate complex to form a developed complex. It is the streptavidin portion of the enzyme complex which binds to the biotinylated portion of the intermediate complex to form the developed complex. The enzyme portion of the streptavidin/enzyme complex may include various types of enzymes capable of binding with streptavidin to form this complex. Preferred streptavidin/enzyme complexes include streptavidin-alkaline phosphatase and streptavidin-horseradish peroxidase which are available from many commercial sources. An effective amount of the streptavidin/enzyme complex is about 0.1  $\mu\text{g}$  of the enzyme complex per ml of substrate buffer solution, preferably about 0.1  $\mu\text{g}$  to 1  $\mu\text{g}$  of the enzyme complex per ml of the phosphate buffer solution. An effective



amount of time to incubate the streptavidin/enzyme complex on the solid carrier is at least about 30 minutes, and preferably about 1 to 8 hours.

After the streptavidin/enzyme complex has been applied to the solid carrier for an effective amount of time, an effective amount of a chromogenic substrate is applied to the solid carrier until a visible end product or plaque is produced. The visible end product or plaque represents a developed complex. When utilizing the enzyme alkaline phosphatase, such chromogenic substrates such as 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium may be used to effect the color change. When utilizing horseradish peroxidase enzyme, such chromogenic substrates such as 1,4-p-phenylenediamine free base (PPD) or 3-amino-9-ethylcarbazole (AEC) can be used.

After the immunoglobulin secreting cells are incubated on the solid carrier and the developing agents have been applied, a physician or researcher can determine the presence of the allergen-specific immunoglobulin-E. Developed complexes indicate the areas where allergen-specific immunoglobulin-E has bound to the allergens immobilized on the solid carrier.

Another variation of the ELISA technique involves utilizing an enzyme which is directly conjugated to an anti-human IgE antibody. The enzyme/anti-human IgE complex can be applied in effective amounts to the solid carrier for an effective amount of time to permit the enzyme/anti-human IgE complex to bind to the allergen/allergen-specific IgE complexes which may be present to form developed complexes. An effective amount of the enzyme/anti-human IgE complex is at least about 0.1  $\mu$ g of the substrate buffer solution, and preferably about 0.1  $\mu$ g to 1  $\mu$ g per ml of the substrate buffer solution. An effective amount of time to incubate the enzyme/anti-human IgE complex on the solid carrier is at least about 30 minutes, preferably about

one hour to eight hours. In a preferred embodiment, the enzyme/anti-human IgE complex is a horseradish peroxidase/anti-human IgE conjugate or alkaline phosphatase/anti-human IgE conjugate. After the  
5 enzyme/anti-human IgE complex has been incubated on the solid carrier for an effective amount of time, a chromogenic substrate is applied to the solid carrier and will yield a visible end product should the allergen-specific IgE be present. As noted above, when  
10 utilizing the enzyme alkaline phosphatase, such chromogenic substrates such as 5-bromo-4-chloro-3-indolyl phosphate\nitroblue tetrazolium may be used to effect the color change. Such chromogenic substrates such as 1,4-p-phenylenediamine free base (PPD) or 3-  
15 amino-9-ethylcarbazole (AEC) can be used when utilizing the horseradish peroxidase enzyme.

A method for diagnosis of allergies in a patient is carried out by utilizing similar steps for detecting  
20 allergen-specific IgE as noted above. The solid carrier used in this method for diagnosis of allergies preferably has a plurality of wells with each well having unique type of allergen or different types of allergens immobilized thereon. As noted above, each  
25 well should have a surface area of at least about 1.5 cm<sup>2</sup> per ml of the immunoglobulin secreting cell solution applied to each well, preferably having a surface area from about 1.5 cm<sup>2</sup> to 1.76 cm<sup>2</sup>. A surface area of at least about 0.33 cm<sup>2</sup> to 1.76 cm<sup>2</sup> may be used if 0.25 ml  
30 aliquots of the immunoglobulin secreting cell solution are applied to each well. This surface area ensures that all immunoglobulin secreting cells are spatially-separated from each other.

The method of diagnosis of allergies in a patient  
35 and the method of detecting immunoglobulin-E differ with respect to the last step. After the immunoglobulin secreting cells are incubated on the solid carrier and

the developing agents have been applied, the number of developed immunoglobulin-E complexes can be determined to ascertain whether an allergic reaction to the specific allergen has occurred on the solid. The extent of a patient's allergic reaction to an allergen is reflected by the number of developed complexes or plaques detected on the solid carrier, each such developed complex representing a site where allergen-specific immunoglobulin-E has bound to the allergen immobilized on the solid carrier. Generally 0 to 4 developed immunoglobulin-E complexes per  $5 \times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate that no allergic reaction has occurred. Most animal systems utilizing IgE-immunoglobulin secreting cells maintain a basal level of about 0-4 immunoglobulin secreting cells per  $5 \times 10^6$  peripheral blood leukocytes at any one time. About 5 to 15 developed immunoglobulin-E complexes detected per  $5 \times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate a marginal allergic response. About 16 to 25 developed immunoglobulin-E complexes per  $5 \times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate a slight allergic reaction. About 26 to 35 developed immunoglobulin-E complexes per  $5 \times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate a moderate allergic reaction. In excess of 35 developed immunoglobulin-E complexes produced per  $5 \times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate a severe allergic reaction. These categories are summarized in Table I below.

TABLE I

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5	5 x 10 <sup>6</sup> peripheral blood cells added to the solid carrier	
10	Number of developed immunoglobulin-E <u>complexes</u>	<u>Extent of Allergic Reaction</u>
	0-4	None
	5-15	Marginal allergic reaction
	16-25	Slight allergic reaction
15	26-35	Moderate allergic reaction
	36-or more	Severe allergic reaction

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20

The number of developed immunoglobulin-E complexes depends on the number of peripheral blood leukocytes applied to the solid carrier. A physician or researcher can modify Table I based on the number of peripheral blood leukocytes applied to the solid carrier. For example, if 1 x 10<sup>7</sup> peripheral blood leukocytes are applied to the solid carrier, the number of developed immunoglobulin-E complexes to indicate a certain allergic response will be doubled. For example, 51 to 70 developed immunoglobulin-E complexes per 1 x 10<sup>7</sup> peripheral blood leukocytes applied to the solid carrier indicate a moderate allergic response.

The present invention also involves a method for diagnosing the presence of allergies in a patient who is not exhibiting allergic symptoms. The method for diagnosing IgE in patients having dormant allergies involves a variation of the method as described hereinabove. The method as disclosed hereinbelow involves an ex vivo provocation test utilizing the ASEPT™ test. The test allows diagnosis of allergies in a patient without subjecting that patient directly to the allergens. This test, therefore, eliminates the

risk that a patient will develop a severe allergic reaction because it is an ex vivo test. This method of diagnosis also permits assessing the efficacy of immunotherapy.

5 A physician or researcher utilizing the ex vivo provocation test will first draw blood from a patient suspected of having a dormant allergy. The blood is then processed by using known techniques to separate blood leukocytes from other blood components. These  
10 peripheral blood leukocytes are then resuspended in biocompatible aqueous solution, preferably fetal calf serum, which preserves the viability of the peripheral blood leukocytes. The solution containing the peripheral blood leukocytes may then be delivered in  
15 various doses, preferably in 1.0 ml doses.

The peripheral blood leukocytes are then applied to a solid carrier having concentrated amounts of one or more different types of allergens mobilized thereon as noted above. It is also necessary that at least about  
20  $1.0 \times 10^5$  peripheral blood leukocytes are applied to the solid carrier to ensure that IgE can be detected should one of these cells transform into an IgE immunoglobulin-secreting cell. The solid carriers utilized in this variation have allergens immobilized thereon and are  
25 identical to those solid carriers disclosed above.

After the peripheral blood leukocytes have been applied to the solid carrier, they are incubated at about 37°C in a 5% to 10% CO<sub>2</sub> plastic module on the solid carrier for a sufficient amount of time to allow the  
30 cells to grow and transform into immunoglobulin-secreting cells. This incubation period should last at least about 72 hours. A 72-hour incubation period stimulates the peripheral blood leukocytes to transform into immunoglobulin-secreting cells and allows these  
35 immunoglobulin-secreting cells a sufficient amount of time to secrete detectible amounts of immunoglobulin. Preferably, the peripheral blood leukocytes are



incubated on the solid carrier about 72 to about 120 hours. The IgE produced after the prolonged incubation period can be developed with the developing agents and quantified pursuant to the disclosure as disclosed above.

The present invention also involves a diagnostic kit for detecting the presence of allergies in a patient. The kit will include at least one solid carrier having concentrated amounts of at least one type of allergen immobilized thereon. The solid carrier may be defined by a plurality of wells, each having a surface area of at least about 1.5 cm<sup>2</sup> for each 1 ml of aqueous solution of peripheral blood leukocytes, preferably each has a surface area of about 1.5 cm<sup>2</sup> to about 1.76 cm<sup>2</sup> per 1 ml of aqueous solution of peripheral blood leukocytes. This surface area ensures that the immunoglobulin secreting cells will be spatially-separated when applied to the well. Each well may have a unique type of allergen or allergens immobilized on the solid carrier. This will enable a physician or researcher to test a patient's immunoglobulin secreting cells against a variety of different allergens.

The diagnostic kit will also include diagnosing agents capable of detecting the presence of allergen-specific IgE produced as a result of the patient's allergic reaction to the concentrated allergen on the carrier. As noted above, a developing agent is any agent capable of reacting with an allergen/allergen-specific immunoglobulin-E complex on the solid carrier to produce a developed and detectable immunoglobulin-E complex. The physician or researcher can then determine whether an allergic reaction to the specific allergen has occurred. The physician or researcher may then compare the number of developed immunoglobulin-E complexes with an indicator, such as a card displaying, for example, the information contained in Table I as previously disclosed. Other indicator cards displaying

information based on other specific numbers of immunoglobulin secreting cells applied to the solid carrier may also be included in the kit for convenience.

The following examples further illustrate specific embodiments of the invention. The examples, however, are not meant to limit the scope of the invention which has been fully characterized in the foregoing disclosure.

### Example 1

10

#### Materials and methods

##### Animals

Two-month old female Lou/cN rats were obtained from Dr. Richard Smith, Case Western Reserve University. The rats were raised and maintained by Dr. Smith throughout the study. CAF<sub>1</sub> and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME).

15

##### Immunization

The rats were immunized twice with 10 µg of mouse monoclonal anti-DNP IgE (Liu et al., J. Immunol., 124, 2728, 1980; Chen et al., J. Exp. Med., 160, 953, 1984) incomplete Freund's adjuvant, 10 µg of IgE soluble IgE in vivo. Rats were rested and boosted with 100 µg of IgE in 4 mg Al(OH)<sub>3</sub> i.p. and later with 50 µg of soluble IgE in vivo. Spleen cells of immunized Lou rats were fused with P3X63Ag 8.653 plasmacytoma immunoglobulin nonsecretor cells obtained from the Salk Institute. The plates were coated with 50 µl of different classes of monoclonal or myeloma immunoglobulins at 1 µg/ml.

Supernatants from confluent hybridoma cultures were diluted one to five, tested for activities against each immunoglobulin isotype, and developed with alkaline phosphatase coupled to goat anti-rat IgG (AP-GARaG).

20

25

30

Female CAF<sub>1</sub> mice were primed with 1 µg keyhole limpet hemocyanin (KLH) in 2 mg Al(OH)<sub>3</sub> i.p. 7 days later, mice were boosted with 1 µg KLH in 2 mg Al(OH)<sub>3</sub>. One group of mice received three daily injections of cyclosporin A



(CsA) at 150 mg/kg i.m., prior to antigen priming. This protocol was shown to optimally induce antigen-specific IgE responses as described in Chen, Transpl. Proc., 22 (suppl. 2), 92, 1988 and Chen et al., J. Immunol., 142, 4225, 1989.

#### Reagents

Monoclonal anti-dinitrophenol (DNP) IgM, IgG1, IgG2b, IgE and anti-ragweed (RAG) IgE were affinity purified as described in Liu et al., J. Immunol., 124, 2728, 1980 and Chen et al., J. Exp. Med., 160, 953, 1984. Murine anti-NP IgE hybridoma 91.58 (Bose et al., Immunology, 53, 81, 1984) was obtained from Dr. E. Rector of the University of Winnipeg, and was purified on an nitrophenol-borine-serum-albumin-Sepharose 4B column. Rabbit anti-mouse DNP-specific IgE (RAME) and goat anti-mouse DNP-specific IgE (GAME) hyperimmune sera were prepared as described in Liu et al., J. Immunol., 124, 2778, 1980 and Chen et al., J. Exp. Med., 160, 957, 1984, and rendered IgE isotype specific by sequential affinity adsorption on Sepharose 4B columns coupled with normal mouse sera. IgG and IgA, and finally adsorbed and eluted from monoclonal anti-RAG IgE-Sepharose 4B column. Pure myeloma immunoglobulins MOPC 315( $\lambda$ ,  $\alpha$ ), MOPC21( $\kappa$ ,  $\gamma_1$ ), MOPC195( $\kappa$ ,  $\gamma_{2b}$ ), and RPC20 ( $\lambda_2$ ) were obtained from Organon Teknika (Malveran, PA). Rat IgG was obtained from Axell (Westbury, NY). A panel of rabbit anti-rat immunoglobulin isotype-specific reagents made by Dr. H. Bazin was obtained from Dr. R. Smith, including anti-IgG2a (SLG2a-9), anti-IgG2b (SLG2b-9), anti-IgG2c (SLG2c-5), anti-IgM (SLM1), anti-IgA (SLA1), and anti-IgE (SLE1). EM95 supernatant, a rat anti-mouse IgE monoclonal antibody product, was provided by Drs. M. Baniyash and Z. Eshhar at Weizmann Institute (Baniyash and Eshhar, 1984). Affinity purified goat anti-rat IgG (GARaG), goat anti-mouse IgG1 (GAMG1) and goat anti-mouse IgM (GAM $\mu$ ) were obtained from Southern Biotechnology Associates (SBA, Birmingham, AL), 5-bromo-

4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD), p-nitrophenyl phosphate (PNPP) was obtained from Sigma Co. (St. Louis, MO). Streptavidin-alkaline phosphatase (SA-AP) was obtained from Zymed (San Francisco, CA). Alkaline phosphatase-swine anti-goat IgG (AP-SAGG), and lactoperoxidase was obtained from Boehringer-Mannheim (Indianapolis, IN). Biotin long chain N-hydroxy succinimide ester (biotin-X-NHS) was obtained from Calbiochem (La Jolla, CA). Dulbecco minimal essential medium (D-MEM) was obtained from M.A. Bioproducts (Walkersville, MD). Fetal calf serum was obtained from HyClone Laboratories (Logan, UT).

15 Biotinylation of rat monoclonal antibodies

Mab anti- $\epsilon$  from clone B 12 or BF 815 was affinity purified by rabbit anti-rat IgG coupled Sepharose 4B and extensively dialysed in a phosphate buffer solution (PBS) at pH 7.8 without azide, 4  $\mu$ l of biotin-X-NHS at 10 mg/ml in 100% dimethylsulfoxide (DMSO) was added to 100  $\mu$ l of MAb anti- $\epsilon$  at 3 mg/ml in PBS. The reaction mixture was left at 4°C for 12 hours and dialyzed against PBS.

ELISA-plaque assay (EP)

25 Nitrocellulose (NC) membrane (0.45  $\mu$ M pore size, 47 mm diameter) was obtained from MFS (Dublin, CA). The membrane was pasted with photomount from 3M (St. Paul, MN) in 60 times 15 mm tissue culture plates (Falcon 3002) directly, or cut in 15 mm in diameter and pasted to the 24-well Linbro plates. NC discs were prewetted in PBS for 1 minute and incubated with 0.3 ml of antigen at 100  $\mu$ g/ml for 30 minutes at room temperature. The wells were rinsed and blocked with Blotto from Carnation Co. (Los Angeles, CA) for 30 minutes at room temperature, rinsed with PBS twice, and incubated with cells in 0.5 ml D-MEM/10% FCS for 4-6 hours at 37°C in a 10% CO<sub>2</sub> incubator. Cyclohexamide was added at 10  $\mu$ g/ml

to inhibit de novo protein synthesis in antigen-coated control wells as shown in Czerkinsky et al., J. Immunol. Methods, 110, 29, 1988. Cells were then removed from the discs by rinsing 5 times in PBS, and the plates were  
5 incubated with 0.1 ml of biotinylated MAb anti- $\epsilon$  overnight at 4°C. The wells were washed, and incubated with streptavidin-alkaline phosphates for 1 hour at room temperature followed by addition of 5-bromo-4-choloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT)  
10 substrate for 30 minutes at room temperature. The reaction was stopped by rinsing the plates, and the paper discs were left dry. Round blue plaques were easily visualized without magnification.

#### Results

15 Cyclosporin A (CsA) was shown to optimally stimulate Ag-specific IgE responses in Chen, Transpl. Proc., 22 (suppl. 2), 92, 1988 and Chen et al., J. Immunol., 142, 9225, 1989. CAF<sub>1</sub> mice were pretreated with CsA and primed with 1  $\mu$ g KLH in 2 mg alum. KLH primed cells  
20 were cultured in Marbrook vessels, and boosted with antigens. Cell were harvested after 7 days and overlaid onto NC discs coated with KLH antigen in 24 wells. Antigen-specific IgE responses can be enumerated at the single cell level by the ELISA plaque assay.  
25 Antigen-specific IgE, secreted by the plasma cell, formed a concentric diffusion gradient, and was efficiently captured by the antigen absorbed to the NC discs. Biotinylated monoclonal rat anti-mouse IgE (BF815) reacted with IgE bound to NC solid phase, and  
30 the dye deposit resulted from a reaction of BCIP/NBT substrate and conjugate of streptavidin-alkaline phosphatase (SA-AP) bound to biotinylated MAb anti- $\epsilon$ . Nonantigen-specific IgE plasma cells, stimulated by interleukin-4 (IL-4) were detected by NC discs coated  
35 with BF815 (Fig. 1(1)); and antigen stimulation lead to detection of more antigen-specific plasma cells on NC discs coated with KLH antigen (Fig. 1(2) vs. 1(4)).

Figure 1(2) illustrates an ongoing antigen-specific IgE response. Figure 1(4) is an antigen boosted antigen-specific IgE response.

The IgE isotype specificity of EP $\epsilon$  was ascertained. As shown in Table II, GAME blocked 82% of KLH-specific IgE responses and MAb anti- $\epsilon$ : BF815 inhibited 70% of IgE responses but neither anti-isotype affected KLH-specific IgG1 responses: likewise GAMG1 inhibited 58% of IgG1 responses, but not IgE responses, and GAM $\mu$  affected neither IgE nor IgG1 responses. Antigen-specific IgE was synthesized de novo by IgE-secreting plasma cells; and preformed cytophilic IgE on lymphocytes did not interfere with the plasma cell assay, since cyclohexamide added at 10  $\mu$ g/ml during the assay completely abrogated the formation of EP $\epsilon$  (data not shown).

TABLE II  
EFFECT OF ANTI-ISOTYPES OF Ag-SPECIFIC ANTI-BODY  
PRODUCING CELLS ASSAYED BY EP

5  $5 \times 10^6$  immune spleen cells were stimulated with KLH at 1  $\mu\text{g}/\text{ml}$ . Cells were harvested on day 7 after antigen stimulation. For IgE assay, one half of the cells from each culture was added to antigen-coated NC discs and  
10 incubated in the presence of goat anti-mouse isotypes at 2-20  $\mu\text{g}/\text{ml}$  or BF815 at 20  $\mu\text{g}/\text{ml}$  for 4 h at 37°C in a 10%  $\text{CO}_2$  incubator. For IgG1 assay, 1/100 of the cells from each culture were added to antigen-coated NC discs, and incubated with different anti-isotypes as above.  
15 Biotinylated BV815 or GAMG1 at 1  $\mu\text{g}/\text{ml}$  was added to plates overnight at 4°C for developing antigen-specific  $\text{EP}_\epsilon$  or  $\text{EP}_{\gamma 1}$ . The rest of the procedures were performed as described in the materials and methods section.

	Incubation of anti-isotype specific antibodies		Number of EP	
			$\text{EP}_\epsilon$	$\text{EP}_\gamma$
		None	86	9800
25	GAME	2 $\mu\text{g}/\text{ml}$	64	10200
		20 $\mu\text{g}/\text{ml}$	16	12100
	BF815	20 $\mu\text{g}/\text{ml}$	26	11000
	GAMG1	20 $\mu\text{g}/\text{ml}$	88	4200
	GAM $\mu$	20 $\mu\text{g}/\text{ml}$	84	9500

35 The antigen dose-response curve was shown in Fig. 2. Optimal KLH-specific IgE responses were observed in cultures stimulated with 1  $\mu\text{g}/\text{ml}$  KLH for 48 hours; and  
40 the magnitude of total IgE responses assessed on MAb anti- $\epsilon$  coated NC discs was comparable to that of antigen-specific IgE responses observed in KLH coated discs. Fig. 3 showed the kinetics of appearance of IgE-secreting cells. Antigen-specific  $\text{EP}_\epsilon$  rose  
45 significantly on day 5, peaked on day 7, and steeply declined on day 10 after antigen stimulation (Fig. 3,2); these antigen-specific IgE producers apparently were also detected by a total  $\text{EP}_\epsilon$  assay on NC discs coated with BF 815 (Fig. 3B). The augmented total IgE plaque

responses observed on day 5 may be due to bystander activation of IgE B cells of undetermined specificities during the process of antigen stimulation.

The EP $\epsilon$  assay appears to be far superior to regular IgE ELISA in quantitating Ag-specific IgE in Ag-stimulated cell cultures. As shown in Table III, antigen-specific EP $\epsilon$  increased 3.3-5.8-fold in cultures stimulated with antigens for 2 days, whereas the level of total IgE in antigen-stimulated cultures was not significantly different from control cultures (Table III: groups 2, 3, vs. 1). Likewise, antigen-specific EP $\epsilon$  increased 5-7.2-fold in cultures stimulated with antigen from day 0 to day 7, whereas total IgE increase only 2.1-2.5-fold to that of control (Table III: page groups 4,5, vs. 1). Although MAb anti- $\epsilon$  based antigen capture assay detected monoclonal anti-DNP IgE in the standard assay, this assay failed to consistently detect antigen-specific IgE despite that there was 3.3- or 5-fold increase of IgE-secreting cells by EP $\epsilon$  assay (Table III: groups 2 and 5). These antigen-IgE complexes may either fail to bind biotinylated antigens, or may be cleared more rapidly; however, washing antigens from cultures day 2 after antigen stimulation did not improve detection of IgE by the antigen capture assay (Table III: groups 2, 3 vs. 4, 5); moreover, the performance of this assay was not improved by depleting cell supernatants of IgG via adsorption to protein G-Sepharose 4B, or by increasing the dosage of coating MAb anti- $\epsilon$  (data not shown).



TABLE III  
COMPARISON OF IGE RESPONSES DETECTED BY DIFFERENT METHOD OF IMMUNOASSAYS

CAF<sub>1</sub> mice were primed with three bi-weekly i.p. injections of 1 µg KLH in 2 mg Al(OH)<sub>3</sub>. 5x10<sup>6</sup> spleen cells were initiated in Marbrook vessels, and stimulated with KLH at 100 ng or 1 µg/ml for 2 days or 7 days. Cells were harvested on day 7 after initiation of cultures. Antigen-Specific IGE plasma cells were enumerated by Epe assay: antigen-specific IGE in the culture supernatants was determined by antigen capture ELISA with wells coated with BF815. Total IGE sandwich assay was performed with BF815 and b-GAME.

Group	Antigen stimulation(duration)	Ag-specific Epe	Ag-specific IGE(O.D.)	Total IGE(ng/ml)
1	None (background)	32	0.117	142
2	KLH. 100 ng/ml (d0-d2)	104	0.086	104
3	KLH. 1 µg/ml (d0-d2)	185	0.220	195
4	KLH. 100 ng/ml (d0-d7)	229	0.189	355
5	KLH. 1 µg/ml (d0-d7)	159	0.077	215



**Example 2**

Pure phospholipase (PLA<sub>2</sub>), an allergenic component in honey bee venom, was obtained from Sigma Co. (St. Louis, MO). BALB/c mice were injected i.p. with 10 µg PLA<sub>2</sub> in 2 µg alum seven times at weekly intervals. Mice were sacrificed 14 days after the last injection; 5x10<sup>6</sup> spleen cells were cultured in Marbrook chamber, and stimulated with PLA<sub>2</sub> from 0.01 µg/ml to 1.0 µg/ml. On Day 7, after antigen stimulation, cells were harvested and assayed for PLA<sub>2</sub>-specific IgE plaques. The assay was performed in 24-well NC plates coated with 0.5 ml of 100 µg PLA<sub>2</sub>/ml; and the rest of the procedures were performed as described in Chen., J. Immunol. Meth. 135, 129, 1990. As shown in Figure 4, approximately 18 to 30 ASEP<sub>PLA<sub>2</sub></sub> per 5x10<sup>6</sup> cells were observed per Marbrook vessel.

**Example 3**

Ovalbumin (OVA), a food allergen in egg, was obtained from Sigma Co. BALB/c mice were injected with 10 µg OVA in 2 mg alum i.p. four times at weekly intervals. Mice were sacrificed two weeks after the last injection. 5x10<sup>6</sup> spleen cells were cultured in Marbrook vessel, and stimulated with OVA from 0.001 µg to 1 µg/ml. The assay was performed in 24-well NC plates coated with 0.5 ml of 100 µg OVA/ml, and the rest of the procedure was performed as described in Example 2. As shown in Figure 5, approximately 85-105 ASEP<sub>OVA</sub> was observed in cultures stimulated with 0.01 µg and 1 µg OVA/ml.

30

**Example 4**

BALB/c mice were intranasally injected with 10<sup>8</sup> Sendai virus (SV), a parainfluenza virus which causes IgE-mediated allergic reaction in the respiratory tract. Six months later, mice were boosted i.p. with 10 µg viral protein extract prepared in 2 mg alum (Gr 1); alternatively, mice were rechallenged i.p. with 10 µg SV

in 2 mg alum seven days after the initial boosting (Gr 2). Five days after primary or secondary boosting, ASEP<sub>sv</sub> were assessed in Peyer's patches (PP), mesenteric lymph node cells (MLN), and spleens (SPC) on NC plates 5 coated with 0.5 ml of 10 µg/ml viral protein extract, and the rest of the procedure was performed as described in Example 2. The results are shown in Figure 6.

## WHAT IS CLAIMED IS:

1. A method of detecting immunoglobulin-E secreting cells comprising:
  - 5 (a) applying a number of immunoglobulin secreting cells onto a solid carrier having concentrated amounts of one or more allergens immobilized thereon, wherein;
    - (i) the number of immunoglobulin secreting cells is sufficient to deliver immunoglobulin-E  
10 secreting cells onto the solid carrier;
      - (ii) substantially all of the immunoglobulin secreting cells are spatially-separated from each other;
    - (b) incubating the immunoglobulin secreting cells on the solid carrier for a sufficient amount of time to  
15 permit the immunoglobulin secreting cells to produce immunoglobulins, wherein;
      - (i) substantially all the immunoglobulin secreting cells produce diffusion gradients of a specific immunoglobulin around themselves without  
20 interference from immunoglobulins secreted by surrounding cells, some of the immunoglobulin secreting cells being capable of producing diffusion gradients of allergen/allergen-specific immunoglobulin-E complexes;
        - (ii) the allergens immobilized on the solid  
25 carrier bind the diffusion gradients to form allergen/immunoglobulin complexes, some of the allergen/immunoglobulin complexes being allergen/allergen-specific immunoglobulin-E complexes;
      - (c) applying a developing agent capable of  
30 detecting the allergen/allergen-specific immunoglobulin-E complexes to the solid carrier to produce a developed complex; and
      - (d) determining the presence of the allergen/allergen-specific immunoglobulin-E on the solid  
35 carrier.

2. A method of detecting immunoglobulin-E secreting cells according to claim 1, wherein the number of immunoglobulin secreting cells is at least about  $1.0 \times 10^4$  immunoglobulin secreting cells/ml.

5

3. A method of detecting immunoglobulin-E secreting cells according to claim 2, wherein the number of immunoglobulin secreting cells is about  $1.0 \times 10^4$  to about  $5.0 \times 10^7$  immunoglobulin secreting cells/ml.

10

4. A method of detecting immunoglobulin-E secreting cells according to claim 3, wherein the number of immunoglobulin secreting cells is about  $1.0 \times 10^6$  to  $1.0 \times 10^7$  immunoglobulin secreting cells/ml.

15

5. A method of detecting immunoglobulin-E secreting cells according to claim 1, wherein the solid carrier has a plurality of wells, each having a surface area of at least about  $1.5 \text{ cm}^2$  to allow the immunoglobulin secreting cells to be spatially-separated from each other.

6. A method of detecting immunoglobulin-E secreting cells according to claim 1, wherein the sufficient amount of time to permit the immunoglobulin secreting cells to produce immunoglobulins is at least about two hours.

7. A method of detecting immunoglobulin-E secreting cells according to claim 6, wherein the sufficient amount of time to permit the immunoglobulin secreting cells to produce immunoglobulins is about two to six hours.

8. A method of detecting immunoglobulin-E secreting cells according to claim 1, wherein the concentrated

amount of allergens immobilized on the solid carrier is at least about 1 micrograms allergen/cm<sup>2</sup>.

9. A method of detecting immunoglobulin-E secreting  
5 cells according to claim 8, wherein the concentrated amount of allergens immobilized on the solid carrier is about one to ten micrograms allergen/cm<sup>2</sup>.

10. A method of detecting immunoglobulin-E secreting  
10 cells according to claim 1, wherein the allergens are selected from a group consisting of microbial allergens, flora allergens, fauna allergens, chemical allergens and any combination thereof.

15 11. A method of detecting immunoglobulin-E secreting cells according to claim 1, wherein the solid carrier is comprised of nitrocellulose.

12. A method of detecting immunoglobulin-E secreting  
20 cells according to claim 1, wherein the step of combining the developing agent comprises:

(a) applying an effective amount of an enzyme/anti-human immunoglobulin-E complex onto the solid carrier for an effective amount of time to permit the  
25 enzyme/anti-human immunoglobulin-E complex to bind to the allergen/allergen-specific immunoglobulin-E complexes to form the developed complex; and

(b) applying an effective amount of a chromogenic substrate to a solid carrier such that a visible colored  
30 end product is produced in the presence of the developed complex bound to the solid carrier.

13. A method of detecting immunoglobulin-E secreting cells according to claim 12, wherein the effective  
35 amount of enzyme/anti-human immunoglobulin-E is about 1.0 µg of enzyme/anti-human immunoglobulin-E per ml of phosphate buffer solution.

14. A method of detecting immunoglobulin-E secreting cells according to claim 12 wherein said enzyme/anti-human immunoglobulin-E complex comprises a horseradish  
5 peroxidase/anti-human immunoglobulin-E conjugate.

15. A method of detecting immunoglobulin-E secreting cells according to claim 14 wherein said chromogenic substrate is selected from the group consisting of 1,4-  
10 p-phenylenediamine free base and 3-amino-9-ethylcarbazole.

16. A method of detecting immunoglobulin-E secreting cells according to claim 12 wherein said enzyme/anti-  
15 human immunoglobulin-E complex comprises an alkaline phosphate/anti-human immunoglobulin-E conjugate.

17. A method of detecting immunoglobulin-E secreting cells according to claim 16 wherein said chromogenic  
20 substrate is selected from the group consisting of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium;

18. A method of detecting immunoglobulin-E secreting  
25 cells according to claim 1, wherein the step of combining the developing agent comprises:

(a) applying an effective amount of a biotinylated anti-human immunoglobulin-E complex onto the solid  
carrier for an effective amount of time to permit the  
30 biotinylated anti-human immunoglobulin-E complex to bind to the allergen/allergen-specific immunoglobulin-E complexes to form an intermediate complex;

(b) applying an effective amount of a streptavidin/enzyme complex for an effective amount of  
35 time to permit the streptavidin/enzyme complex to bind to the intermediate complex to form the developed complex; and

(c) applying an effective amount of a chromogenic substrate to a solid carrier such that a visible colored end product is produced in the presence of the developed complex bound to the solid carrier.

5

19. A method of detecting immunoglobulin-E secreting cells according to claim 18, wherein said streptavidin/enzyme complex comprises a streptavidin-alkaline phosphatase conjugate.

10

20. A method of detecting immunoglobulin-E secreting cells according to claim 18, wherein said streptavidin/enzyme complex comprises a streptavidin horseradish peroxidase conjugate.

15

21. A method of detecting immunoglobulin-E secreting cells according to claim 20, wherein said chromogenic substrate is selected from the group consisting of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue

20 tetrazolium; 1,4-p-phenylenediamine free base; and 3-amino-9-ethylcarbazole.

22. A method for diagnosis of allergies:

(a) applying a sample of a patient's peripheral  
25 blood leukocytes onto a solid carrier having concentrated amounts of at least one allergen immobilized thereon, wherein;

(i) the sample of peripheral blood leukocytes is sufficient to deliver immunoglobulin-E secreting  
30 cells onto the solid carrier;

(ii) substantially all of the immunoglobulin secreting cells are spacially separated from each other;

(b) incubating the patient's immunoglobulin secreting cells on the solid carrier for a sufficient  
35 amount of time to permit the immunoglobulin secreting cells to produce immunoglobulins, wherein;



(i) substantially all the immunoglobulin secreting cells produce diffusion gradients of a specific immunoglobulin around themselves without interference from immunoglobulins secreted by surrounding cells, some of the immunoglobulin secreting cells being capable of producing diffusion gradients of allergen-specific immunoglobulin-E;

(ii) the allergens immobilized on the solid carrier bind the diffusion gradients to form allergen/immunoglobulin complexes, some of the allergen/immunoglobulin complexes being allergen/allergen-specific immunoglobulin-E complexes;

(c) applying a developing agent capable of detecting the allergen/allergen-specific immunoglobulin-E complexes to the solid carrier to produce a developed immunoglobulin-E complex; and

(d) determining whether the number of the developed immunoglobulin-E complexes is sufficient to indicate the presence of an allergy in the patient.

20

23. A method of diagnosis of allergies according to claim 22, wherein the solid carrier has multiple allergens immobilized thereon.

25 24. A method diagnosis of allergies according to claim 22, wherein the solid carrier has a plurality of wells, each having a surface area of at least about  $1.5 \text{ cm}^2$  to allow the immunoglobulin secreting cells to be spacially separated from each other.

30

25. A method of diagnosis of allergies according to claim 24, wherein each well has at least about one type of allergen immobilized thereon, each specific well has a unique type of allergen immobilized thereon.

35

26. A method of diagnosis of allergies according to claim 22, wherein the allergens are selected from a

group consisting of microbial allergen, flora allergens, fauna allergens, chemical allergens and any combination thereof.

5 27. A method of diagnosis of allergies according to claim 22, wherein the step of determining whether the number of the developed immunoglobulin-E complexes is sufficient to indicate the presence of an allergy in the patient comprises counting each developed  
10 immunoglobulin-E complex present on the solid carrier.

28. A method of diagnosing the presence of an allergy according to claim 27, wherein

(a) 0-4 developed immunoglobulin-E complexes per 5  
15  $\times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate that no allergic reaction has occurred;

(b) 5 to 15 developed immunoglobulin-E complexes per 5  $\times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate that a possible allergic reaction  
20 has occurred;

(c) 16-25 developed immunoglobulin-E complexes per 5  $\times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate that a slight allergic reaction has occurred;

25 (d) 26-35 developed immunoglobulin-E complexes per 5  $\times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate that a moderate allergic reaction has occurred; and

(e) 36 or more developed immunoglobulin-E complexes  
30 per 5  $\times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate that a severe allergic reaction has occurred.

29. A method for diagnosing allergies in a patient who  
35 is not exhibiting allergic symptoms comprising:

(a) applying a number of peripheral blood leukocytes from the patient onto a solid carrier having

concentrated amounts of one or more allergens immobilized thereon, wherein;

(i) the number of peripheral blood leukocytes is sufficient to ensure that immunoglobulin-E can be  
5 detected should one or more peripheral blood leukocytes transform into an immunoglobulin-E secreting cell;

(ii) substantially all of the peripheral blood leukocytes are spatially separated from each other;

(b) incubating the peripheral blood leukocytes on  
10 the solid carrier for a sufficient amount of time to allow the cells to transform to immunoglobulin secreting cells and to allow these immunoglobulin secreting cells to secrete detectable amounts of immunoglobulin, wherein;

(i) substantially all the immunoglobulin  
15 secreting cells produce diffusion gradients of a specific immunoglobulin around themselves without interference from immunoglobulins secreted by surrounding cells, some of the immunoglobulin secreting  
20 cells being capable of producing diffusion gradients of allergen/allergen-specific immunoglobulin-E complexes;

(ii) the allergens immobilized on the solid carrier bind the diffusion gradients to form an allergen/immunoglobulin complexes, some of the  
25 allergen/immunoglobulin complexes being allergen/allergen-specific immunoglobulin-E complexes;

(c) combining a developing agent capable of detecting the allergen/allergen-specific immunoglobulin-E complexes on the solid carrier to produce a developed  
30 complex; and

(d) determining the presence of the allergen/allergen-specific immunoglobulin-E on the solid carrier.

35 30. A method for diagnosing allergies in a patient who is not exhibiting allergic symptoms according to claim

29, wherein the number of peripheral blood leukocytes is at least about  $1.0 \times 10^4$  peripheral blood leukocytes/ml.

31. A method for diagnosing allergies in a patient who is not exhibiting allergic symptoms according to claim 30, wherein the number of peripheral blood leukocytes is about  $1.0 \times 10^4$  to  $5.0 \times 10^7$  peripheral blood leukocytes/ml.

32. A method for diagnosing allergies in a patient who is not exhibiting allergic symptoms according to claim 30, wherein the number of peripheral blood leukocytes is about  $1.0 \times 10^6$  to  $1.0 \times 10^7$  peripheral blood leukocytes/ml.

33. A method for diagnosing allergies in a patient who is not exhibiting allergic symptoms according to claim 29, wherein a sufficient amount of time to allow the cells to transform to immunoglobulin cells and to allow these immunoglobulin secreting cells to secrete detectable amounts of immunoglobulin is at least about 72 hours.

34. A method for diagnosing allergies in a patient who is not exhibiting allergic symptoms according to claim 29, wherein a sufficient amount of time to allow the cells to transform to immunoglobulin cells and to allow these immunoglobulin secreting cells to secrete detectable amounts of immunoglobulin is at least about 72 to 120 hours.

35. A diagnostic kit for detecting the presence of allergies in a patient comprising:

- (a) a solid carrier having concentrated amounts of at least about one allergen immobilized thereon; and
- (b) diagnosing reagents capable of detecting the presence of allergen-specific immunoglobulin-E produced.

as a result of a patient's allergic reaction to the concentrated allergen on the solid carrier.

36. A diagnostic kit according to claim 35, wherein the  
5 diagnosing reagents comprises:

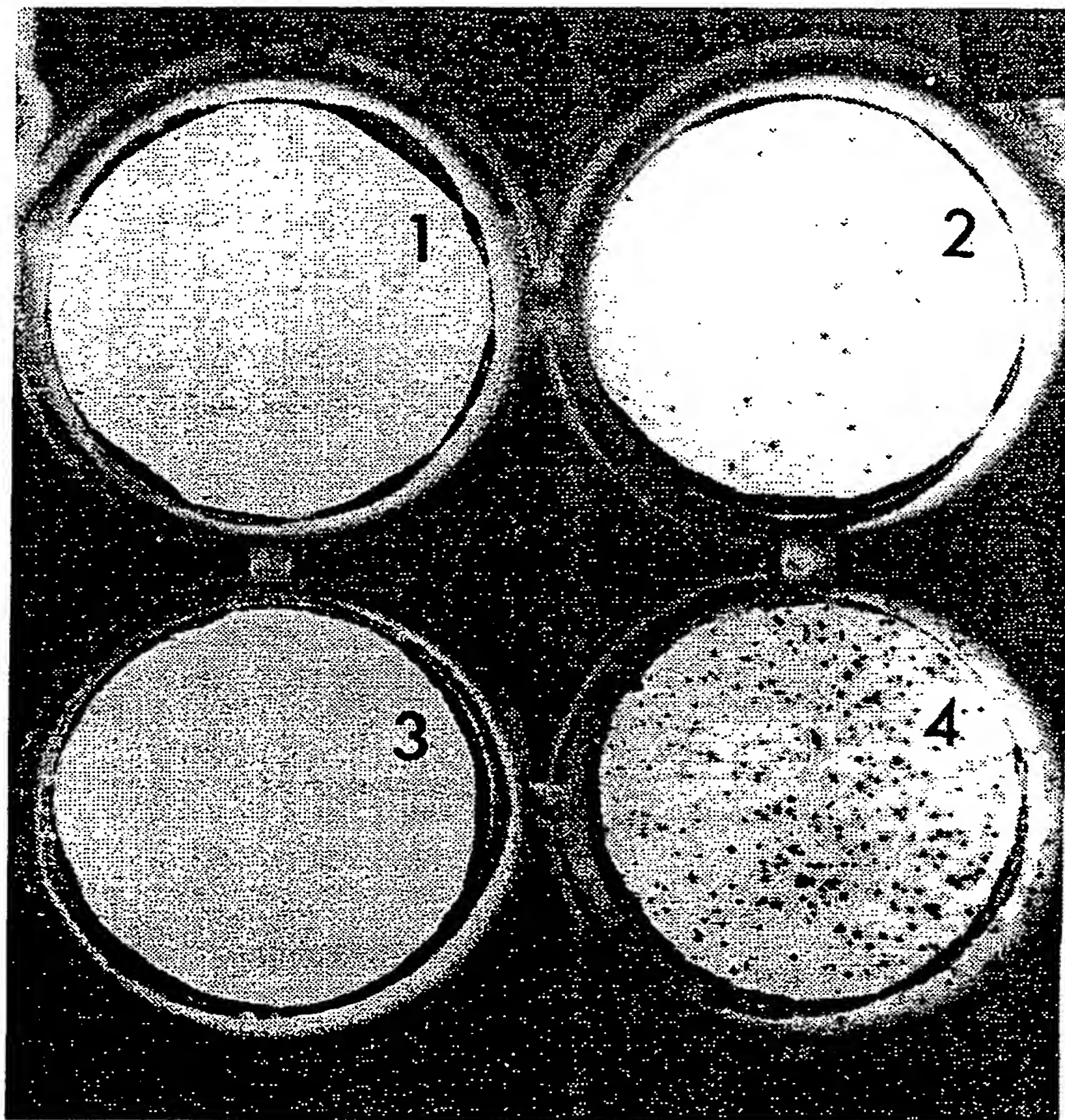
- (a) a developing agent capable of reacting with an allergen/allergen-specific immunoglobulin-E complexes on the solid carrier to produce a developed immunoglobulin-E complex; and
- 10 (b) a quantifying material capable of determining the number of allergen-specific immunoglobulin-E complexes produced on the solid carrier.

37. A diagnostic kit according to claim 36, wherein the  
15 quantifying material is an indicator displaying that

- (a) 0-4 developed immunoglobulin complexes on the solid carrier per  $5 \times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate that no allergic response to the allergen on the solid carrier has  
20 occurred;
- (b) 5-15 developed immunoglobulin-E complexes on the solid carrier per  $5 \times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate a possible allergic reaction to the allergen immobilizes on the  
25 solid carrier;
- (c) 16-25 developed immunoglobulin-E complexes on the solid carrier per  $5 \times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate a slight allergic reaction to the allergen immobilized on the solid  
30 carrier;
- (d) 25-35 developed immunoglobulin-E complexes on the solid carrier per  $5 \times 10^6$  peripheral blood leukocytes applied to the solid carrier indicate a moderate allergic reaction to the allergen immobilized on the  
35 solid carrier; and
- (e) 36 or more immunoglobulin-E complexes on the solid carrier per  $5 \times 10^6$  peripheral blood leukocytes

applied to the solid carrier indicate a severe allergic reaction to the allergen immobilized on the solid carrier.





**FIG. I**

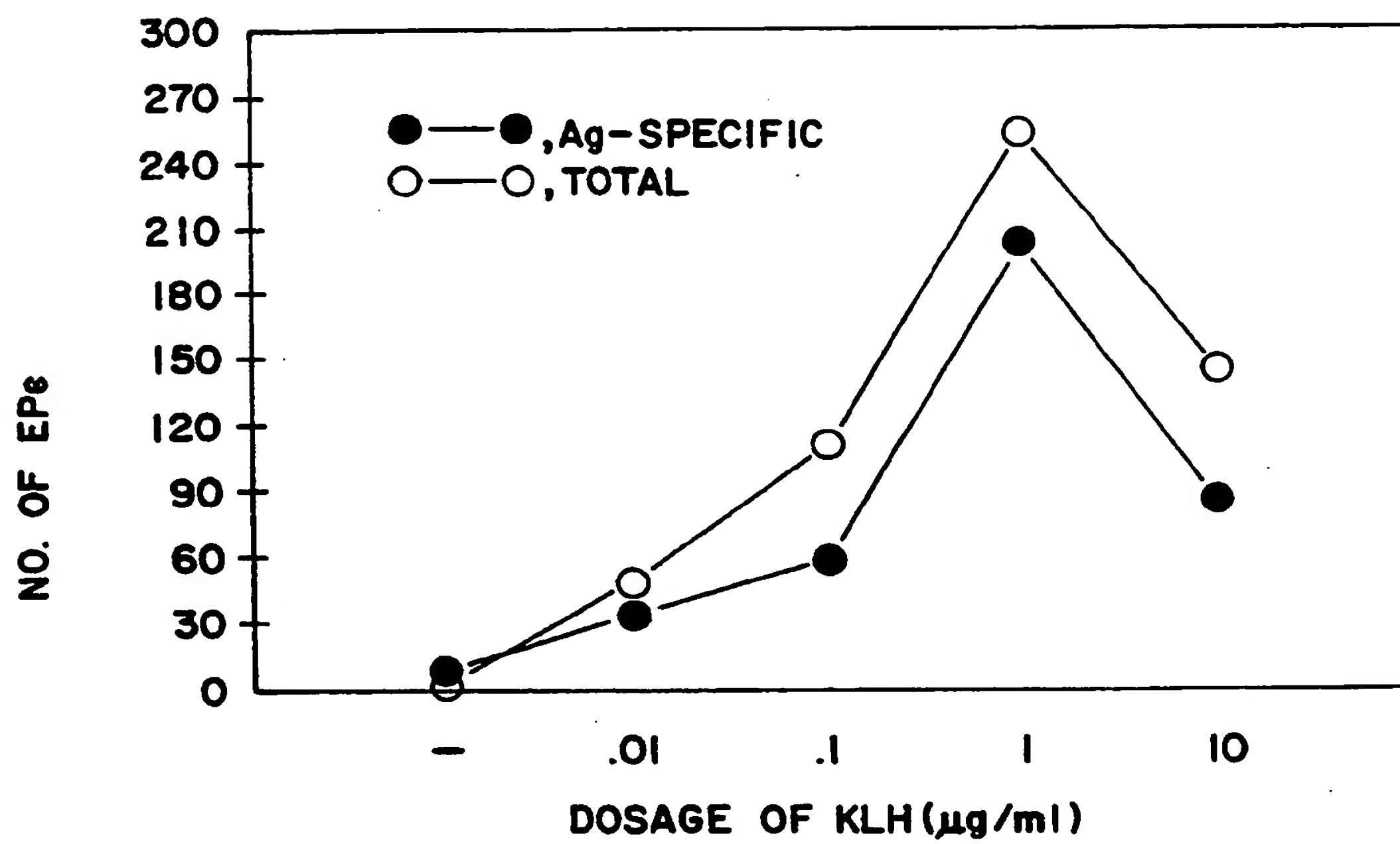


FIG. 2

FIG. 3A

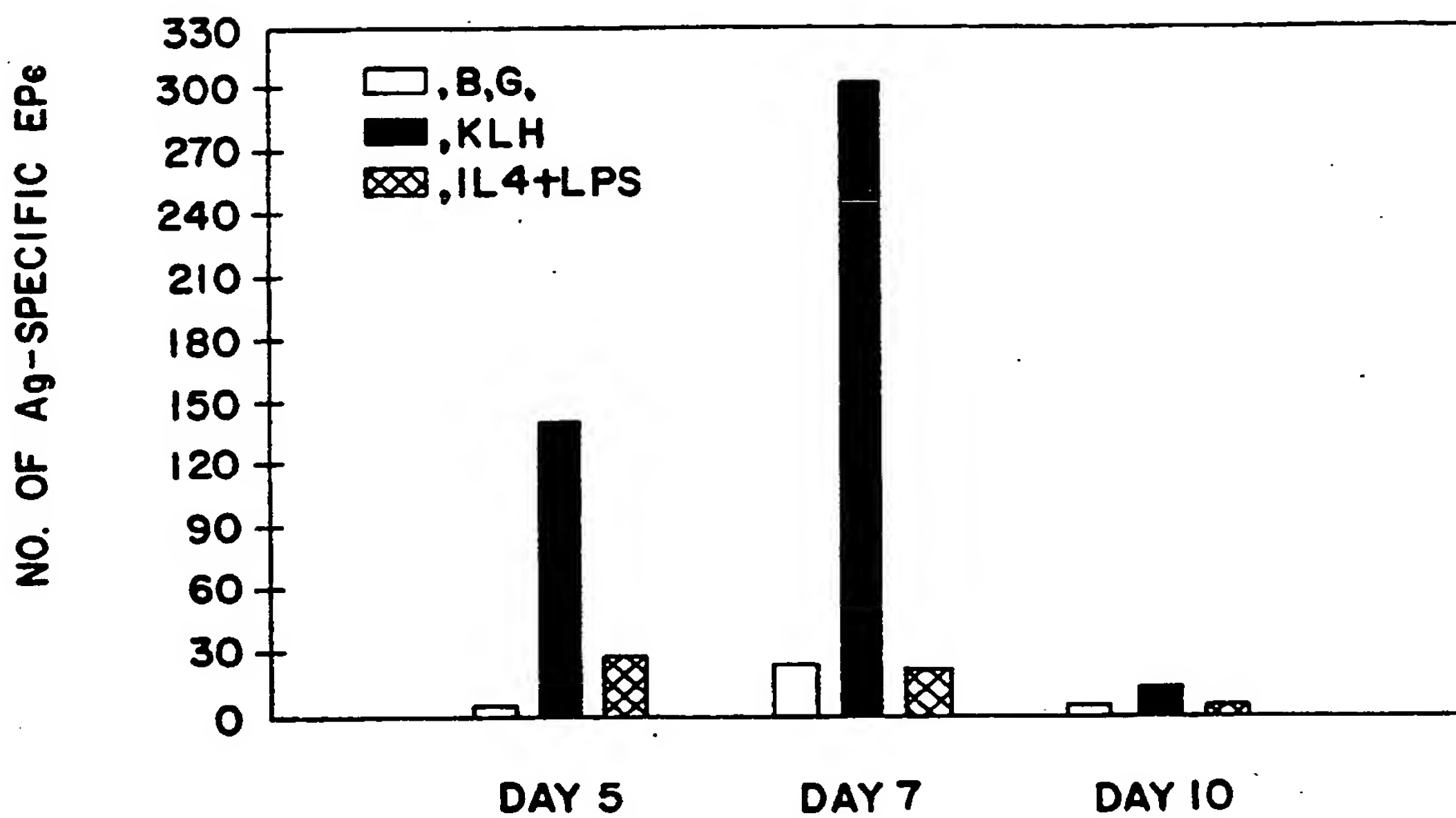
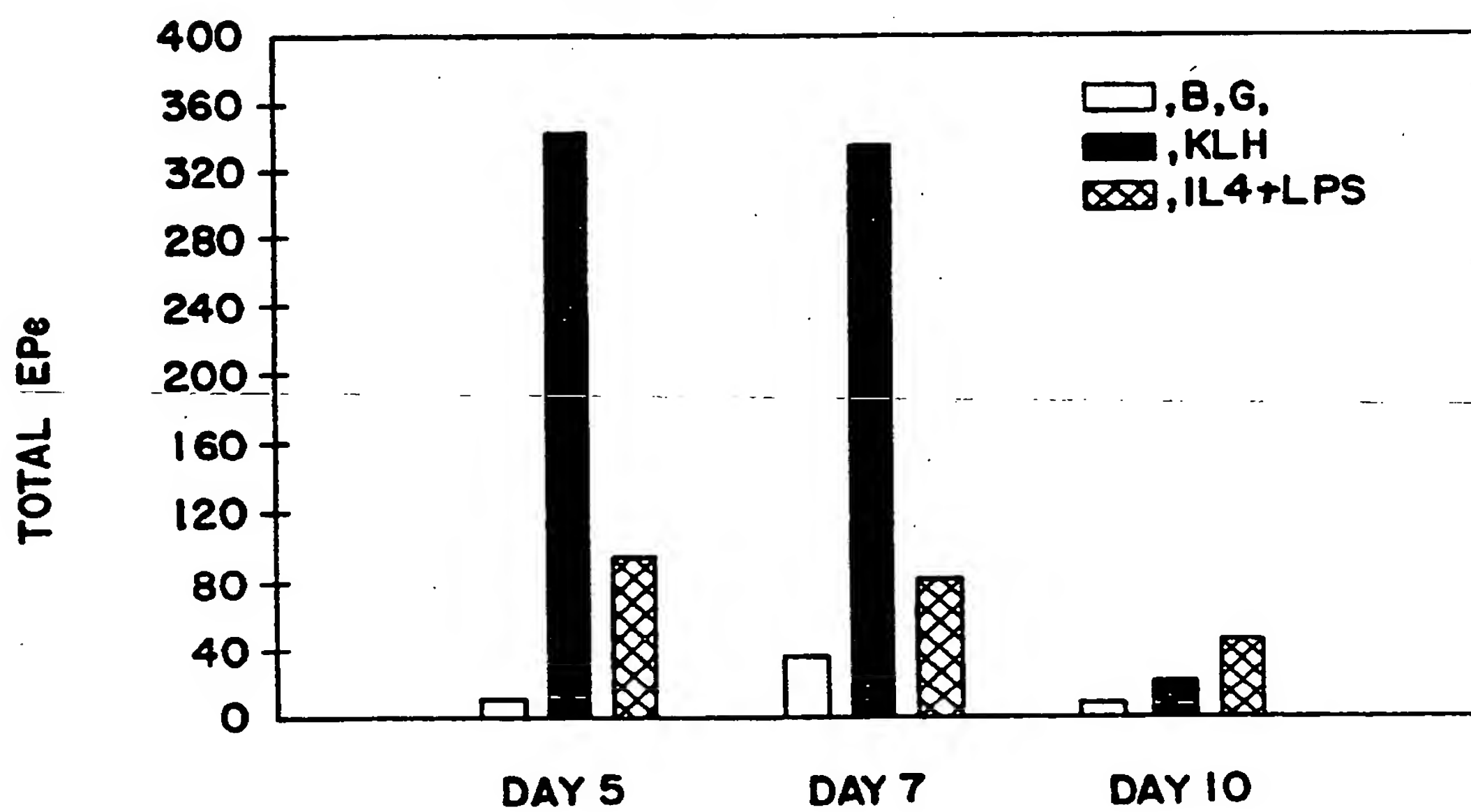


FIG. 3B



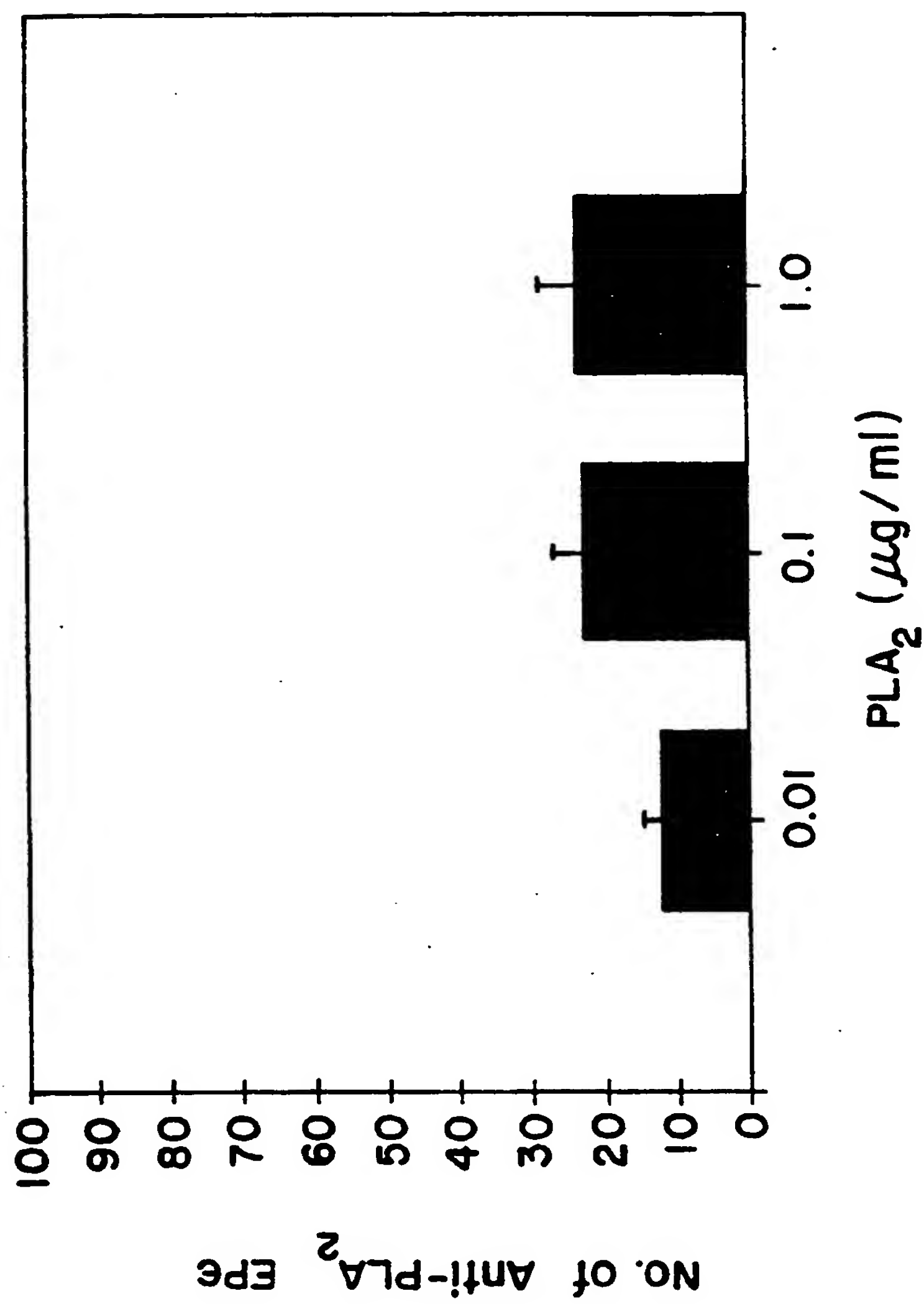


FIG. 4

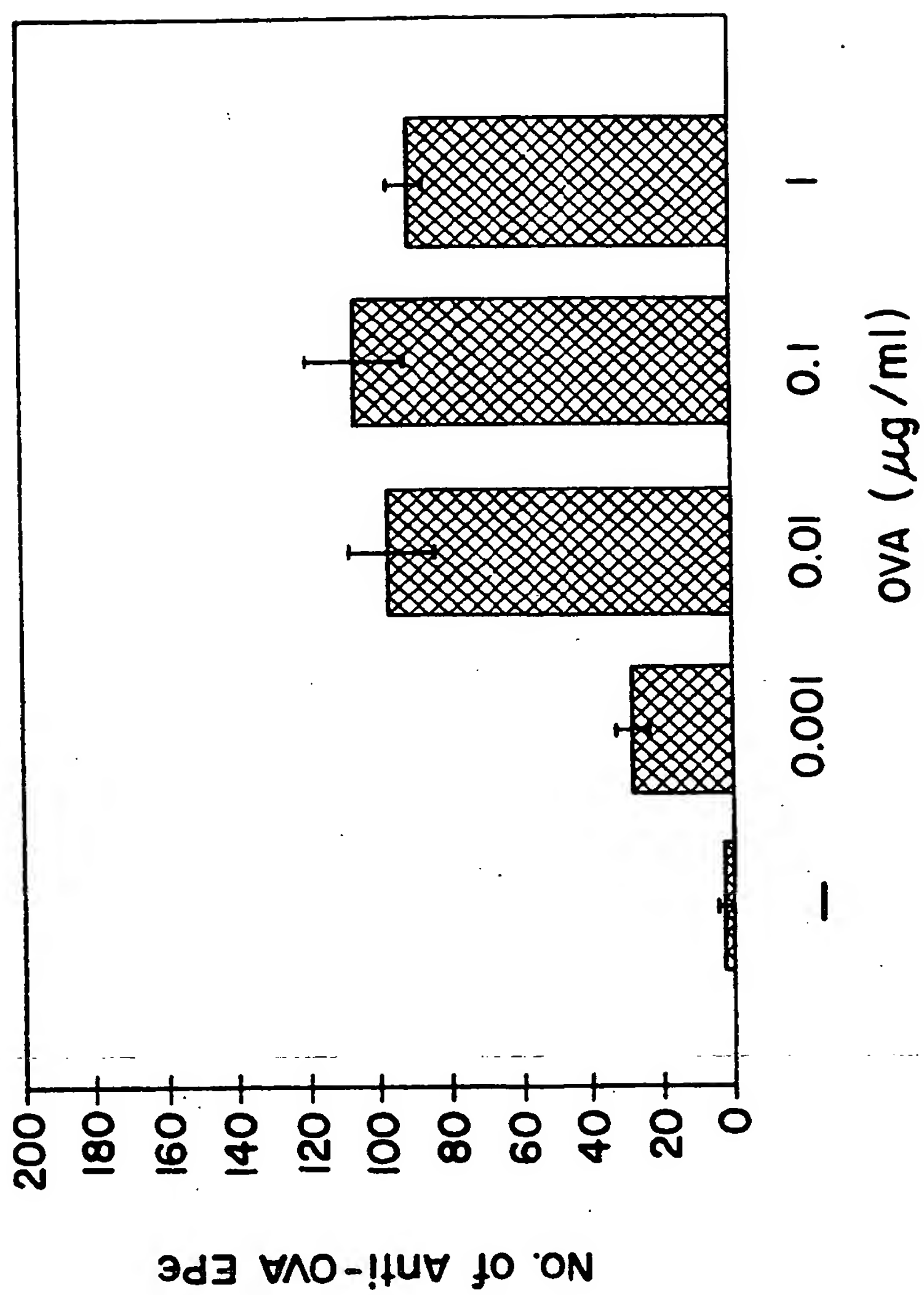


FIG. 5

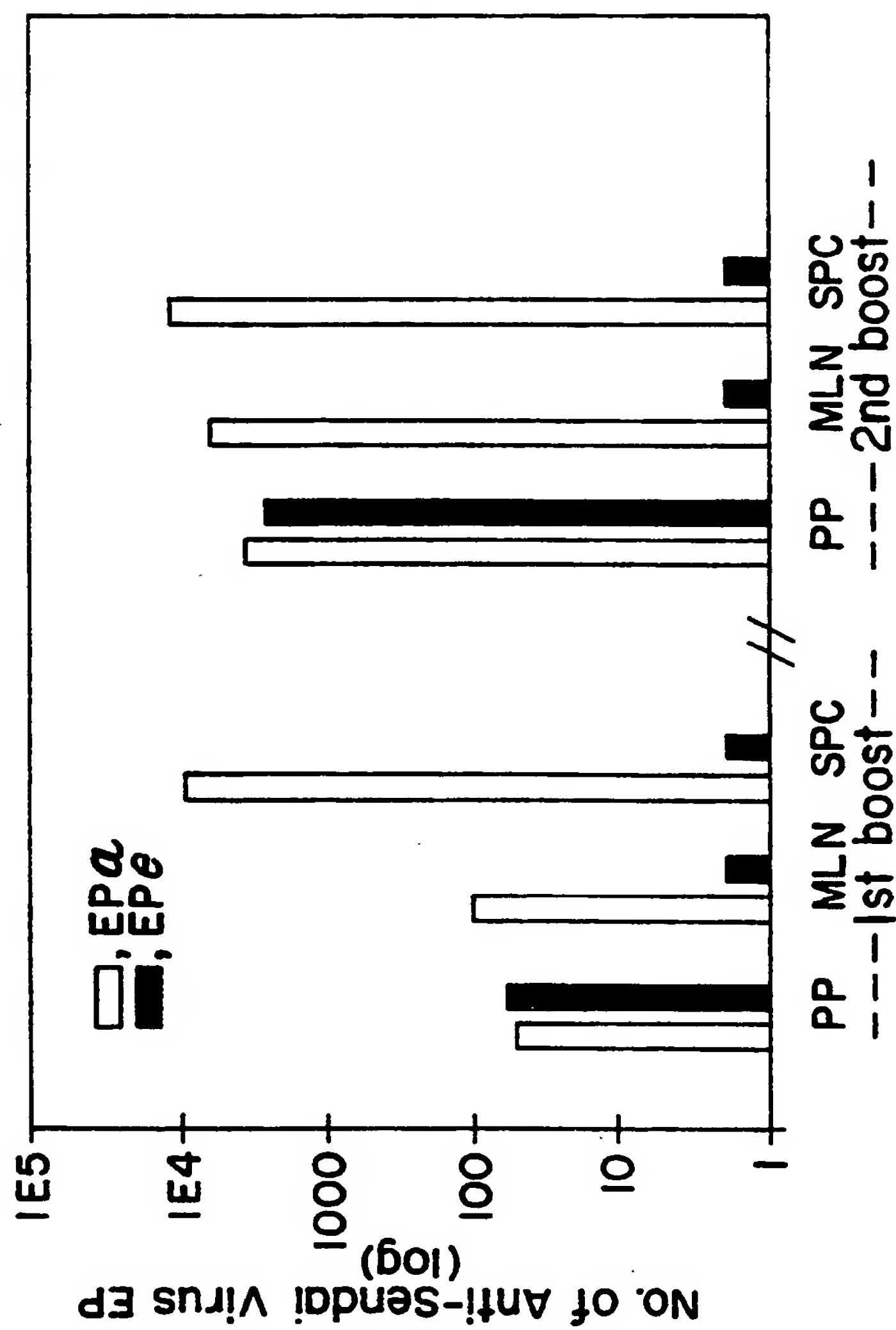


FIG. 6



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/09416

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 G01N33/68; G01N33/569; G01N33/558; G01N33/543		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	G01N ; C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP,A,0 396 505 (CIBA-GEIGY AG) 7 November 1990	1-37
Y	see page 9, line 56 - page 11, line 3; claims 1,31-35,37; example 8.1.2 ---	1-37
Y	EP,A,0 119 613 (MINNESOTA MINING AND MANUFACTURING COMPANY) 26 September 1984 see the whole document ---	1-37
Y	EP,A,0 451 800 (ABBOTT LABORATORIES) 16 October 1991 see the whole document ---	1-37
Y	EP,A,0 219 461 (MARCUCCI, FRANCESCO) 22 April 1987 see the whole document ---	1-37
		-/--
<sup>10</sup> Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 12 FEBRUARY 1993		Date of Mailing of this International Search Report 25.02.93
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer DÖPFER K.P.

Form PCT/ISA/210 (second sheet) (January 1985)

## III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
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A	DE,A,3 147 763 (STICKL, HELMUT) 9 June 1983 see the whole document ---	1-37
A	BIOLOGICAL ABSTRACTS vol. 72, no. 5 , 1981, Philadelphia, PA, US; abstract no. 28495, BENVENISTE, J. 'The human basophil degranulation test as an in vitro method for the diagnosis of allergies' see abstract & CLIN. ALLERGY 11(1)1-12. 1981 -----	

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US 9209416  
SA 66677

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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